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RESEARCH ARTICLE

Overproduction of translation elongation factor 1-α (eEF1A) suppresses the peroxisome biogenesis defect in a *Hansenula polymorpha pex3* mutant via translational read-through

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Abstract

In eukaryotes, elongation factor 1-α (eEF1A) is required during the elongation phase of translation. We observed that a portion of the cellular eEF1A colocalizes with purified peroxisomes from the methylotrophic yeast *Hansenula polymorpha*. We have isolated two genes (*TEF1* and *TEF2*) that encode eEF1A, and which are constitutively expressed. We observed that overproduction of eEF1A suppressed the peroxisome deficient phenotype of an *H. polymorpha pex3-1* mutant, which was not observed in a strain deleted for *PEX3*. The *pex3-1* allele contains a UGG to UGA mutation, thereby truncating Pex3p after amino acid 242, suggesting that the suppression effect might be the result of translational read-through. Consistent with this hypothesis, overexpression of the *pex3-1* gene itself (including its now untranslated part) partly restored peroxisome biogenesis in a *PEX3* null mutant. Subsequent co-overexpression of *TEF2* in this strain fully restored its peroxisome biogenesis defect and resulted in the formation of major amounts of full-length Pex3p, presumably via translational read-through.

Introduction

The eukaryotic translation elongation factor 1-α (eEF1A) is essential for the delivery of aminoacyl-tRNA (aa-tRNA) molecules to the ribosome during the elongation phase of translation (Riis *et al*., 1990; Merrick, 1992). Similar to EF-Tu, its bacterial and mitochondrial counterpart, eEF1A binds aa-tRNA and GTP to form a ternary complex (eEF1A/GTP/aa-tRNA), which delivers the charged aa-tRNA to the A site of the ribosome at the expense of GTP hydrolysis. In addition to its role in translation elongation, eEF1A also appears to control the accuracy of translation. Mutations are known to occur in *Saccharomyces cerevisiae* eEF1A which increase the level of mistranslation and result in frameshifting as well as read-through at stop codons. Furthermore, it was demonstrated that changes in the level of *S. cerevisiae* eEF1A parallel changes in the misreading of nonsense codons (for review see Valente & Kinzy, 2003). Similar effects have been observed in the yeast *Podospora anserina* (Silar & Picard, 1994; Silar *et al*., 2000).

In addition to a role in translation, eEF1A has also been demonstrated to function in multiple other cellular processes (see Lamberti *et al*., 2004) including, among others, apoptosis, ubiquitin-mediated protein degradation, calmodulin binding and actin binding, and bundling. Initially, these additional roles for eEF1A were observed exclusively in higher eukaryotes. However, recently it has been demonstrated that eEF1A is also an actin-binding and bundling protein that affects the actin cytoskeleton in *S. cerevisiae* (Munshi *et al*., 2001). Strikingly, it has been observed that, like eEF1A, actin also has a role in the translational fidelity in baker's yeast (Kandl *et al*., 2002).

Here we show that in the methylotrophic yeast *Hansenula polymorpha* a portion of the cellular eEF1A cofractionates with peroxisomes. Peroxisomes are essential, inducible

organelles present in all eukaryotic cells. These structures have a relatively simple morphology and harbour many enzymes required for highly diverse biochemical pathways (van den Bosch *et al.*, 1992), which also includes methanol metabolism in *H. polymorpha* (recently reviewed by van der Klei *et al.*, 2006). The presence of eEF1A in peroxisome fractions prompted us to study this protein in more detail. The results of this study are included in this paper.

Materials and methods

Strains and media

The *H. polymorpha* strains used in this study are listed in Table 1 and were grown in batch cultures on (1) mineral medium (MM; van Dijken *et al.*, 1976) using glucose (0.5% w/v), ethanol (0.5% v/v), glycerol (0.5% v/v), methanol (0.5% v/v) or glycerol+methanol (0.1%+0.5% v/v, respectively) as carbon sources, and ammoniumsulphate (0.25% w/w) or D-alanine (0.25% w/w) as nitrogen sources; or on (2) rich medium (YPD) containing 1% (w/v) yeast extract, 1% (w/v) peptone and 1% (w/v) glucose. Transformants were selected on YND plates containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 1% (w/v) glucose and 1.5% (w/v) agar. Amino acids, nucleotides (both at a final concentration of 30 $\mu g\,mL^{-1}$) and zeocin (100 $\mu g\,mL^{-1}$) were added as required.

Escherichia coli DH5α and C600 (Sambrook *et al.*, 1989) were used for plasmid constructions and were cultured on (1) LB medium supplemented with the appropriate antibiotics or on (2) M9 medium with the appropriate vitamins, amino acids and nucleotides (Sambrook *et al.*, 1989).

Isolation and analysis of p52 from purified peroxisomes

Peroxisomes were purified from osmotically lysed *H. polymorpha* CBS4732 protoplasts via discontinuous sucrose density gradient centrifugation (Douma *et al.*, 1985), and peroxisomal proteins were separated by SDS-PAGE. A dominant protein of 52 kDa (p52) was isolated using preparative SDS-PAGE. Microsequencing of internal peptides of p52 obtained after trypsin digestion and HPLC separation was performed by EuroSequence (EuroSequence, Groningen, the Netherlands). This resulted in the peptide sequences: p52-33: (Thr)-(Val)-Pro-Phe-Val-Pro-Ile-Ser and p52-31 (Thr)-Leu-Leu-Glu-Ala-Ile-Asp-Ala-Ile-Glu-Pro-Pro-Ala-Arg-Pro-Ser-Asp-Lys-Pro-Leu-Arg-Leu-Pro-Leu-Gln-Asp-Val-Tyr-(Lys), which were highly similar to amino acids 185-192 and 225-254 of the *S. cerevisiae* translation elongation factor 1α (eEF1A; Schirmaier & Philippsen, 1984), respectively.

DNA procedures

Standard DNA techniques were carried out essentially according to Sambrook *et al.* (1989). Transformation of *H. polymorpha* was performed as described previously (Faber *et al.*, 1994). DNA modifying enzymes were used as recommended by the supplier (Roche, Almere, the Netherlands). Southern blot analysis was performed using the ECL direct nucleic acid labeling and detection system (Amersham Corp, Arlington Heights, IL).

Cloning and sequencing of *H. polymorpha TEF1* and *TEF2*

To isolate the gene(s) that encoded p52, we designed degenerate PCR primers based on two internal amino acid

Table 1. *Hansenula polymorpha* strains used in this study

Strain	Genotype	Reference
NCYC495	*leu1.1 ura3*	Gleeson & Sudbery (1988)
CBS4732	Prototrophic	CBS culture collection
tef1	NCYC495 *tef1*::*CaLEU2 ura3*	This study
tef2	NCYC495 *tef2*::*HpURA3 leu1.1*	This study
TEF1-lacZ	NCYC495::P_{TEF1}*TEF1-lacZ CaLEU2 ura3*	This study
TEF2-lacZ	NCYC495:: P_{TEF2}*TEF2-lacZ CaLEU2 ura3*	This study
pex3-1	NCYC495 *pex3-1 leu1.1* (previously designated *per 9-1*)	Baerends *et al.* (1996)
pex3-1 [pHIPX4-TEF2]	*pex3-1* containing plasmid overexpressing the *TEF2* gene	This study
pex3	NCYC495 *pex3::URA3 leu1.1*	Baerends *et al.* (2000)
KEF2-1	*pex3*::(P_{AOX}-*MYC-PEX3-1*ORF)^{mc}	This study
KEF9-6	*pex3*::(P_{AOX}-*MYC-PEX3-1*total)^{mc}	This study
KEF2-1::P_{AOX}-*TEF2*	KEF2-1 with integrated pHIPZ4-TEF2 plasmid	This study
KEF9-6::P_{AOX}-*TEF2*	KEF9-6 with integrated pHIPZ4-TEF2 plasmid	This study
pex3 [pRBG61]	*pex3* with plasmid containing P_{PEX3}-*MYC-PEX3*	Haan *et al.* (2002)

Ca, *C. albicans*; *Hp*, *H. polymorpha*; mc, multicopy.

sequences determined from the purified protein. PCR amplification with the primers Hp52-1 (5′-AAA GGT ACC GTN CCN TTY GTN CCN AT-3′) – based on amino acids Thr-Val-Pro-Phe-Val-Pro-Ile of peptide p52-33 – and Hp52-3 (5′-TCT TCT AGA GGV ARY CTV ARN GGY TTR TC-3′) – based on amino acids Asp-Lys-Pro-Leu-Arg-Leu-Pro of peptide p52-31 – resulted in the amplification of a specific 200-bp fragment from wild-type *H. polymorpha* genomic DNA, which was cloned in pUC19. Sequencing of the PCR fragment revealed high sequence similarity to genes encoding eEF1A. In addition, Southern blot hybridization demonstrated the presence of two genes encoding p52 in the *H. polymorpha* genome. Subsequently, the cloned fragment was used as a probe to isolate the *H. polymorpha TEF1* gene by colony hybridization using a *H. polymorpha* genomic library in plasmid pHRP2 (Faber *et al.*, 1992). Four positive clones were obtained, of which plasmid pKEF3 contained the entire *TEF1* gene. The *TEF2* gene was apparently not present in this gene library, but was shown to be located on a 4.5-kb HindIII genomic *H. polymorpha* fragment by Southern blot hybridization (data not shown). Subsequently, a small gene library was constructed in pUC19 using HindIII-digested *H. polymorpha* genomic DNA (fragment sizes 4–5 kb). A clone containing *TEF2* was identified by colony hybridization and designated as pKEF5.

For the determination of the nucleotide sequence of *TEF1*, subclones were generated in pBluescript II SK^+ (Stratagene Inc., San Diego, CA) that were sequenced using the T7 polymerase system (Pharmacia). For *TEF2* a series of nested deletions was generated by the limited exonuclease III digestion method (Sambrook *et al.*, 1989), and these deletions were sequenced on an ABI 313A automatic sequencer (Applied Biosystems Inc.) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit. In addition, several selected oligonucleotides were synthesized to complete or confirm certain portions of the *TEF1* and *TEF2* DNA sequences. BLAST algorithms (Altschul *et al.*, 1997) were used to screen databases at the National Center for Biotechnology Information (Bethesda, MD). The nucleotide sequences of *H. polymorpha TEF1* and *TEF2* were deposited at GenBank and were assigned the accession numbers AY179868 (*TEF1*) and AY179869 (*TEF2*).

Construction of *TEF1* and *TEF2* null mutants

To disrupt *TEF1*, we constructed plasmid pKEF403, a pUC19 derivative that contains the *Candida albicans LEU2* gene (Genbank accession number AF000121) flanked by the promoter region of *TEF1* (up to the HpaI site at nt 936 of AY179868) and the 3′ end of the *TEF1* coding sequence (downstream from the EcoRV site at nt 1135 of AY179868). This will result in a deletion of the region of *TEF1* encoding amino acids 9 to 74. For the *TEF2* disruption, the HpaI–NcoI (blunted by Klenow treatment) fragment of pKEF5 (nt 794–1388 of AY179869, the region of *TEF2* encoding amino acids 9–207) was replaced by a 2.3-kb BamHI (blunted by Klenow treatment) fragment containing the *H. polymorpha URA3* gene (Merckelbach *et al.*, 1993), resulting in plasmid pKEF504. Subsequently, a 3.6-kb PvuI–PstI fragment of pKEF403 (*TEF1*) or a 2.9-kb PvuII fragment of pKEF504 (*TEF2*) was used to transform *H. polymorpha* NCYC495 (*leu1.1 ura3*) (see Fig. 1). Leucine (*TEF1*) or uracil (*TEF2*) prototrophic transformants were screened by Southern blot analysis for proper disruption (data not shown). Crossing of the *tef1* (*ura3*) and *tef2* (*leu1.1*) strains and random spore analysis of the resulting diploids were performed according to Titorenko *et al.* (1993).

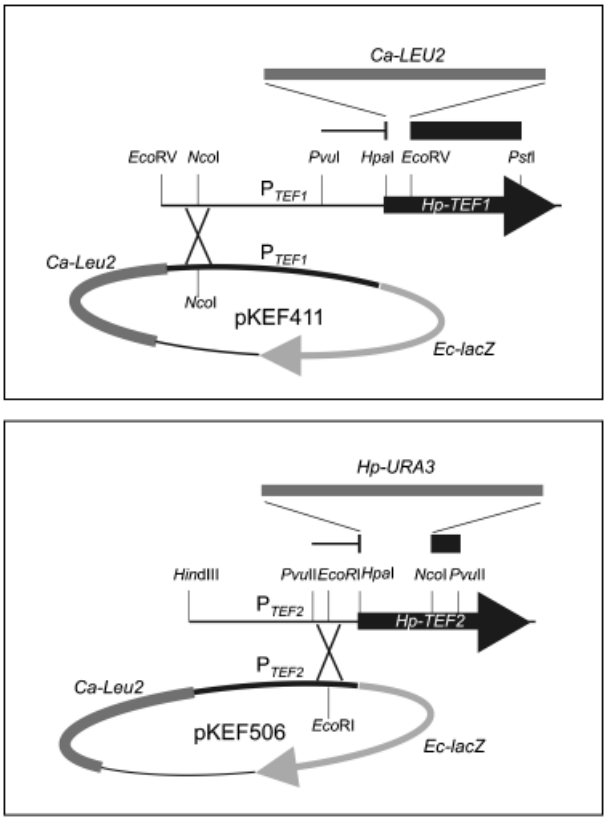

Fig. 1. Schematic representation of the *H. polymorpha* genomic regions containing *TEF1* and *TEF2*. Also indicated are the strategies used to construct the *H. polymorpha tef1* and *tef2* strains as well as the strains carrying integrated copies of the in-frame fusion genes between *TEF1* or *TEF2* and the *E. coli lacZ* gene. Only relevant restriction sites are indicated. *Ca*, *C. albicans*; *Ec*, *E. coli*; *Hp*, *H. polymorpha*.

Construction of *TEF* gene fusions with the *E. coli lacZ* gene

To enable quantitative measurement of the expression levels of *H. polymorpha TEF1* and *TEF2* we constructed plasmids

pKEF411 and pKEF506 harbouring in frame fusion genes between the *TEF1* and *TEF2* genes and the *E. coli lacZ* gene lacking a start codon (from plasmid pMLB1034; Silhavy *et al.*, 1984), respectively. In pKEF411 and pKEF506, *lacZ* was placed downstream from a 2-kb EcoRV–HpaI fragment (*TEF1*) or a 1.4-kb HindIII–HpaI (*TEF2*) fragment comprising the promoter region and the first eight codons of the indicated *TEF* gene. In addition, both plasmids contain the *C. albicans LEU2* gene. To enable integration at the homologous locus in the *H. polymorpha* genome, plasmids pKEF411 and pKEF506 were linearized with NcoI and EcoRI in the *TEF1* and *TEF2* promoter regions, respectively, and transformed into *H. polymorpha* (*leu1.1 ura3*) (Fig. 1). Leucine prototrophic transformants were analysed by Southern blot analysis for proper single-copy integration (data not shown).

Overexpression of *TEF2* in *H. polymorpha*

To enable overproduction of eEF1A in *H. polymorpha*, the entire *TEF2* coding region was placed under the control of the strong inducible *H. polymorpha AOX* promoter. First a HindIII site was introduced upstream of the *TEF2* gene by PCR using primer TEF2-H (5′-TGA AAG CTT CAA AAT GGG TAA AG-3′). After subcloning in pBluescript (Stratagene) – a plasmid designated pB-TEF2-rec – a 2.2-kb HindIII–SmaI fragment containing the entire *TEF2* coding region was inserted between the HindIII and SmaI sites of pHIPX4 (Gietl *et al.*, 1994). The resulting plasmid, designated pHIPX4-TEF2, was transformed into various *H. polymorpha pex* mutant strains.

Generation of polyclonal antibodies against eEF1A

The region comprising amino acids 9 to 459 of *H. polymorpha* eEF1A was produced in *E. coli* as part of a fusion protein with maltose-binding protein (MBP) using the Protein Fusion and Purification System (New England Biolabs, Beverly, MA). To this end, a 3-kb HpaI–HindIII fragment of pKEF5 was cloned between the Asp700I and HindIII sites of the pMAL-c2 vector resulting in plasmid pKEF500. Production and purification of the fusion protein was performed according to the instructions of the supplier of the system. Purified MBP-eEF1A fusion protein was used to immunize a rabbit.

Isolation of the mutant *PEX3* gene from *H. polymorpha pex3-1*

The mutant *PEX3* locus was amplified from the genome of the peroxisome deficient *H. polymorpha* strain *pex3-1* by PCR using primers PER9-1A (5′-AGA GGA TCC CGG GTT CGT TCT CTG TGA TAC-3′) and PER9-1B (5′-GTC GTC GAC GAT ATC TAA TCA GTA TAC ATG C-3′). The resulting 1.45-kb PCR fragment was digested with BamHI and SalI and inserted in BamHI+SalI-digested pBluescript, resulting in plasmid pB-pex3-1. Subsequently, two independently isolated clones were sequenced.

Overexpression of mutant versions of *PEX3* in a *H. polymorpha PEX3* null mutant

To overexpress MYC-tagged versions of the *pex3-1* gene in a *H. polymorpha* mutant lacking *PEX3*, we constructed plasmids pX4-MYC-pex3-1ORF and pX4-MYC-pex3-1total. For pX4-MYC-pex3-1ORF, we digested plasmid pRBG60 – a derivative of pRBG61 (Haan *et al.*, 2002) containing the wild-type *MYC-PEX3* gene in the vector pHIPX4 – with HindIII and SmaI, and inserted a 0.75-kb HindIII–EcoRV fragment of pB-pex3-1. This fragment exclusively contains the 242 aa ORF of the *pex3-1* gene. For pX4-MYC-pex3-1total, we inserted the entire mutant *pex3-1* allele from pB-pex3-1 as a 1.35-kb HindIII–SalI fragment between the HindIII and SalI sites of pRBG60. Both plasmids were subsequently integrated in the P_{AOX} locus of the genome of the *H. polymorpha PEX3* null mutant. Proper integration of the plasmids was confirmed by Southern blotting (data not shown). Strains with multiple copies of the expression cassettes in their genomes (designated KEF2-1 and KEF9-6, respectively) were selected for further study.

To enable co-overexpression of MYC-tagged versions of the *pex3-1* gene and the *TEF2* gene we used plasmid pHIPZ4-TEF2. This plasmid was constructed by inserting a 2.2-kb HindIII–XbaI fragment from pB-TEF2-rec, containing the entire *TEF2* gene, between the HindIII and XbaI sites of vector pHIPZ4 (Salomons *et al.*, 2000), which contains the *H. polymorpha AOX* promoter and a zeocin resistance gene. Subsequently, pHIPZ4-TEF2 was linearized with Asp718I and integrated into the genomes of *H. polymorpha* KEF2-1 and KEF9-6.

Biochemical techniques

Hansenula polymorpha protoplasts were generated and lysed according to Van der Klei *et al.* (1998). Postnuclear supernatants were loaded on to discontinuous sucrose gradients as described by Douma *et al* (1985). Fractions of gradients were analysed for sucrose concentrations and alcohol oxidase (AO; Verduyn *et al.*, 1984) and cytochrome c oxidase (COX; Douma *et al.*, 1985) activities. Protein concentrations were determined using the Bio-Rad Protein Assay system using bovine serum albumin as a standard. β-galactosidase activity measurements were performed according to Miller (1972) using crude extracts of *H. polymorpha* cells prepared with glass beads as described by Waterham *et al.* (1994). For

Western blots, cell extracts of *H. polymorpha* cells were prepared using the TCA method (Baerends *et al.*, 2000). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed by established procedures. Western blots were decorated with polyclonal antibodies against various *H. polymorpha* proteins.

Electron microscopy

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Waterham *et al.*, 1994). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against selected *H. polymorpha* proteins.

Results

Isolation of the *H. polymorpha TEF1* and *TEF2* genes encoding eEF1A

Hansenula polymorpha cells, subjected to methylotrophic growth conditions, contain many peroxisomes that harbour enzymes involved in the metabolism of methanol: alcohol oxidase (AO) dihydroxyacetone synthase (DHAS) and catalase (CAT) (Veenhuis *et al.*, 1978; for a recent review see van der Klei *et al.*, 2006). AO and DHAS constitute the majority of the proteins present in peroxisomes (Fig. 2). We observed that in addition to these proteins fractions enriched in peroxisomes, obtained by sucrose density centrifugation of homogenized protoplasts, also contain a relatively dominant protein band of *c.* 52 kDa (designated p52). Microsequencing of this protein band identified two peptides with high similarity to eukaryotic eEF1A.

To confirm (partial) colocalization of eEF1A with peroxisomes, we determined eEF1A levels in fractions of sucrose density gradients prepared from a postnuclear supernatant (PNS) of methanol/ammoniumsulphate-grown wild-type cells by Western blotting using an α-EF1A antiserum. To enable this, polyclonal antibodies were raised against an MBP-eEF1A fusion protein, which specifically recognized a protein with an apparent molecular weight of *c.* 52 kDa on Western blots prepared with crude extracts of *H. polymorpha* wild-type cells (cf. Fig. 5). The data shown in Fig. 8 indicate that indeed a portion of eEF1A cofractionated with peroxisomes (Fig. 3; fractions 4–7). However, the bulk of the eEF1A sedimented to lower density fractions that contain other organelles and cytosolic proteins. Thus, in *H. polymorpha* eEF1A apparently has multiple localizations.

In many yeast species eEF1A is encoded by at least two genes (Schirmaier & Philippsen, 1984; Sundstrom *et al.*, 1987, 1990). In order to isolate the gene(s) encoding eEF1A, we designed degenerate primers based on the peptide sequences and obtained a specific *H. polymorpha* DNA fragment by PCR. Sequence analysis demonstrated that the isolated fragment indeed contained part of a *H. polymorpha* gene encoding eEF1A. Southern blot hybridization with genomic DNA of *H. polymorpha* using the cloned PCR fragment as a probe (Fig. 4) revealed that the *H. polymorpha* genome contains two genes encoding eEF1A (*TEF1* and *TEF2*), which were isolated and sequenced.

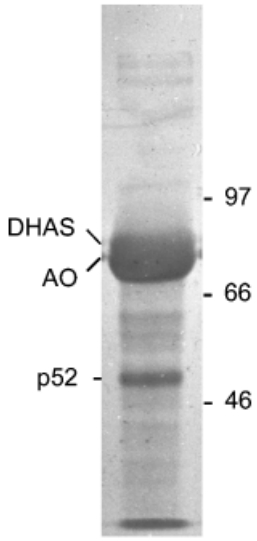

Fig. 2. Identification of *p52*. Proteins present in sucrose density gradient fractions enriched in peroxisomes were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Indicated are the major peroxisomal matrix proteins AO and DHAS as well as the dominant p52 protein. The molecular weights of three marker proteins are indicated in kilodalton to the right.

Analysis of the sequences revealed that *TEF1* and *TEF2* differ only six nucleotides in the region encoding eEF1A (1380 bp). In contrast, the promoter and terminator regions of the genes are rather dissimilar. As was observed for *S. cerevisiae* and *C. albicans* (Schirmaier & Philippsen, 1984; Sundstrom *et al.*, 1990), in *H. polymorpha* the two *TEF* genes encode an identical protein. *Hansenula polymorpha* eEF1A is highly similar to its orthologues from other eukaryotes (81–93% identity) but also shows similarity to prokaryotic EF-Tu proteins (*c.* 30% identity).

Deletion of *H. polymorpha TEF1* and *TEF2*

In order to analyse the effect of deletion of *TEF1* and/or *TEF2* in *H. polymorpha*, we constructed strains *tef1* and *tef2* (see Fig. 1). In eukaryotes, eEF1A is an essential protein (Cottrelle *et al.*, 1985). To investigate this also for *H. polymorpha*, we crossed the *tef1* and *tef2* strains and subjected prototrophic diploids to random spore analysis. Phenotypic analysis of the progeny from these diploids resulted in a *TEF1*/*TEF2*: *TEF1*/*tef2*: *tef1*/*TEF2*: *tef1*/*tef2*

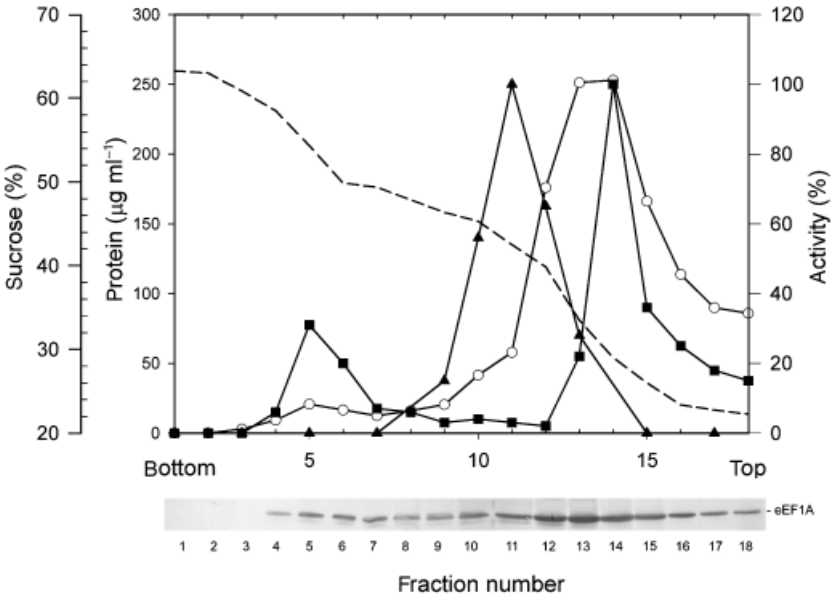

Fig. 3. Biochemical localization of *eEF1A* in *H. polymorpha*. Sucrose gradient, prepared from a postnuclear supernatant of *H. polymorpha* wild-type cells grown on media with methanol/ammoniumsulphate. Sucrose (dashed line), protein concentration (open circles) and the distribution of the activities of the peroxisomal marker alcohol oxidase (closed squares) and the mitochondrial marker cytochrome c oxidase (closed triangles) are indicated. Enzyme activities are expressed as percentages of the activities of the peak fractions, which were set to 100%. The Western blot shows the distribution of eEF1A in the fractions of the gradient. Equal portions of each fraction were loaded per lane.

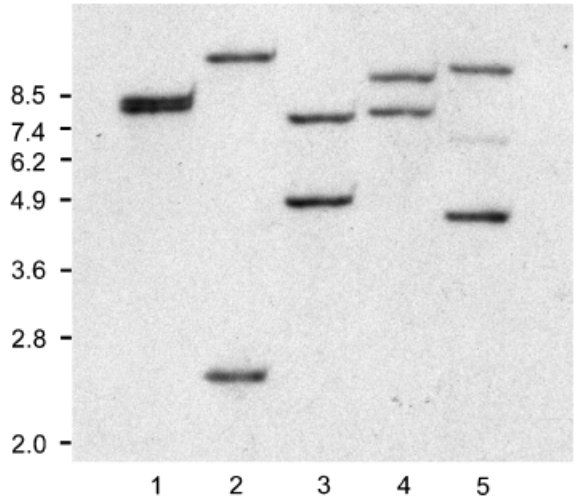

Fig. 4. The *H. polymorpha* genome contains two genes encoding *eEF1A*. Genomic DNA was isolated from *H. polymorpha* CBS4732, digested with BamHI (lane 1), EcoRI (lane 2), HindIII (lane 3), PstI (lane 4) and SalI (lane 5), subjected to agarose gel electrophoresis, blotted and hybridized to an ECL-labeled 200 bp PCR fragment encoding an internal part of *H. polymorpha* eEF1A. Fragment lengths of the marker, phage SPPI DNA restricted with EcoRI, are given in kilobases to the left.

segregation of 1 : 1 : 1 : 0 ($n = 504$), confirming the inviability of a *tef1*/*tef2* double deletion mutant.

We also analysed the effect of the deletion of either *TEF1* or *TEF2* on growth of *H. polymorpha* on medium with different carbon and nitrogen sources. Growth of *tef1* cells was significantly retarded on media containing methanol/ammoniumsulphate or methanol/D-alanine (Table 2). As growth on these substrates involves peroxisomal enzymes, a possible explanation could be that peroxisome formation was affected in *tef1* cells. To study this we performed an ultrastructural analysis of *tef1* cells grown on methanol/D-alanine media, which indicated that the cells contained normal peroxisomes similar to WT controls (data not shown). Furthermore, immunocytochemistry using specific antibodies against AO and CAT showed no significant mislocalization of these peroxisomal matrix proteins in *tef1* cells (data not shown).

Hansenula polymorpha TEF1 and *TEF2* are constitutively expressed genes

The data presented above suggest that *H. polymorpha TEF1* and *TEF2* may not be expressed to the same extent and/or that their expression levels/profiles may depend on the carbon/nitrogen sources used for growth. To understand more about the expression levels/profiles of *TEF1* and *TEF2*, we placed a *TEF-lacZ* fusion gene containing the first eight codons of *TEF1*/*TEF2* fused in frame to the *E. coli lacZ* gene under the control of the genomic *TEF1* and *TEF2* promoters, respectively (see Fig. 1), and determined β-galactosidase activities in crude extracts of cells grown under various conditions. We also determined eEF1A protein levels in *H. polymorpha* wild-type, *tef1* and *tef2* cells by Western blotting using samples taken at different time-intervals during growth on YPD medium. The results of these experiments demonstrate (1) that both *TEF1* and *TEF2* promoters are active on all media tested, with the lowest expression levels on medium with methanol/D-alanine as carbon/nitrogen source (Table 3); (2) that eEF1A is constitutively present during growth of *H. polymorpha* on YPD

Table 2. Growth characteristics of *Hansenula polymorpha* strains on different carbon and nitrogen sources

	Doubling time t_d (h)			Yield ($OD_{663\,nm}$)		
Carbon/nitrogen source	Wild-type	*tef1*	*tef2*	Wild-type	*tef1*	*tef2*
Glucose/NH_4^+	1.6	1.5	1.6	7.2	7.1	7.4
Ethanol/NH_4^+	1.8	1.8	1.9	5.9	5.9	6.1
Glycerol/NH_4^+	2.0	2.1	2.1	5.7	5.8	5.6
Methanol/NH_4^+	3.6	4.1	3.5	5.5	4.0	5.7
Glucose/D-alanine	1.2	1.3	1.1	8.5	7.9	8.6
Methanol/D-alanine	3.4	4.7	3.3	7.7	4.5	7.9

The indicated *H. polymorpha* strains were grown at 43 °C in mineral medium supplemented with various carbon and nitrogen sources. The data represent the average of three independent experiments. The optimal doubling time (t_d) was calculated from growth curves. The yield represents the final $OD_{663\,nm}$ of the cultures.

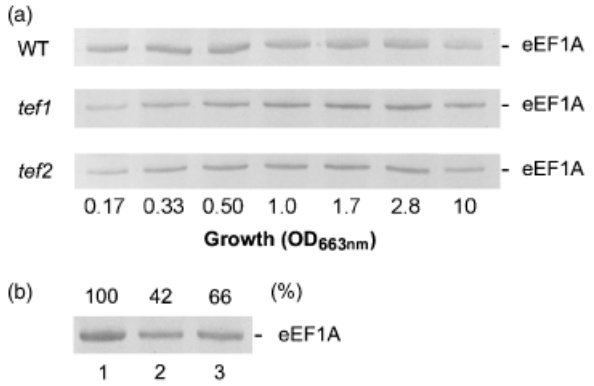

Fig. 5. Steady-state levels of *eEF1A* in *H. polymorpha*. (a) *Hansenula. polymorpha* wild-type, *tef1* and *tef2* cells were pregrown on YPD medium, diluted into fresh YPD medium (to an $OD_{663\,nm}$ of 0.1) and subsequently cultured at 37 °C. Samples were taken during growth at the indicated $OD_{663\,nm}$ values and TCA precipitated. Subsequently, crude cell extracts were prepared and analysed by Western blotting using a specific antiserum against eEF1A. Equal amounts of protein were loaded per lane. (b). *Hansenula polymorpha* wild-type (lane 1), *tef1* (lane 2) and *tef2* (lane 3) cells were grown on YPD medium to mid-exponential growth phase ($OD_{663\,nm}$ = 1.0) and TCA precipitated. Subsequently crude cell extracts were prepared and analysed by Western blotting using a specific antiserum against eEF1A. Equal amounts of protein were loaded per lane. The relative eEF1A levels were determined by densitometric scanning of the indicated Western blot, the value obtained for wild-type being set to 100%.

Table 3. Expression of the *Hansenula polymorpha TEF1* and *TEF2* genes

	β-galactosidase activity (U mg^{-1})			
Strain	Glucose/NH_4^+	Glucose/D-alanine	Methanol/NH_4^+	Methanol/D-alanine
TEF1-lacZ	4120	3120	3010	2630
TEF2-lacZ	2620	1770	1870	1350
Ratio *TEF1*/*TEF2*	1.57	1.76	1.61	1.95

Cells of the indicated *H. polymorpha* strains were cultivated to the mid-exponential growth phase on mineral medium with the indicated carbon and nitrogen sources and β-galactosidase activities were measured in crude cell extracts. No significant β-galactosidase activity (< 50 U mg^{-1}) was observed in wild-type *H. polymorpha* cell extracts. The ratio between the expression levels of *TEF1* and *TEF2* on the various media is also indicated.

medium (Fig. 5a); and (3) that the expression of the *TEF1* gene is *c.* 1.5- to 2-fold higher than that of *TEF2* (Table 3). As a result, extracts of *tef1* cells grown on methanol/D-alanine have the lowest eEF1A levels (Fig. 5b).

Overproduction of eEF1A suppresses the peroxisome biogenesis defect in a *H. polymorpha pex3-1* mutant

Our data indicate that depletion of eEF1A has no effect on peroxisome formation in a wild-type background. We next examined the possibility that overproduction of eEF1A might suppress the peroxisome biogenesis defect of *H. polymorpha pex* mutants defective in peroxisome formation (for an overview of the *PEX* genes present in fungi see Kiel *et al.*, 2006). Overproduction was confirmed by Western blotting using specific antibodies against eEF1A (data not shown). Ultrastructural analysis demonstrated that exclusively in the *pex3-1* mutant (Baerends *et al.*, 1996) partial suppression of the peroxisome biogenesis defect had occurred. Unlike wild-type *H. polymorpha* cells, which contain many peroxisomes (Fig. 6a), peroxisomal structures are not detectable in *pex3-1* cells (Fig. 6b; see also Baerends *et al.*, 1996). Overproduction of eEF1A in *pex3-1* cells resulted in the formation of a single peroxisome in a number of cells (Fig. 6c). Immunocytochemistry demonstrated that this single organelle contained significant amounts of peroxisomal matrix proteins (Fig. 6d, shown for AO).

To understand whether the partial suppression of the peroxisome biogenesis defect in the *pex3-1* mutant was allele-specific, we also investigated whether overproduction of eEF1A could restore peroxisome biogenesis in a *PEX3* null mutant. Ultrastructural analysis of cells of this mutant overexpressing *TEF2* did not show any visible

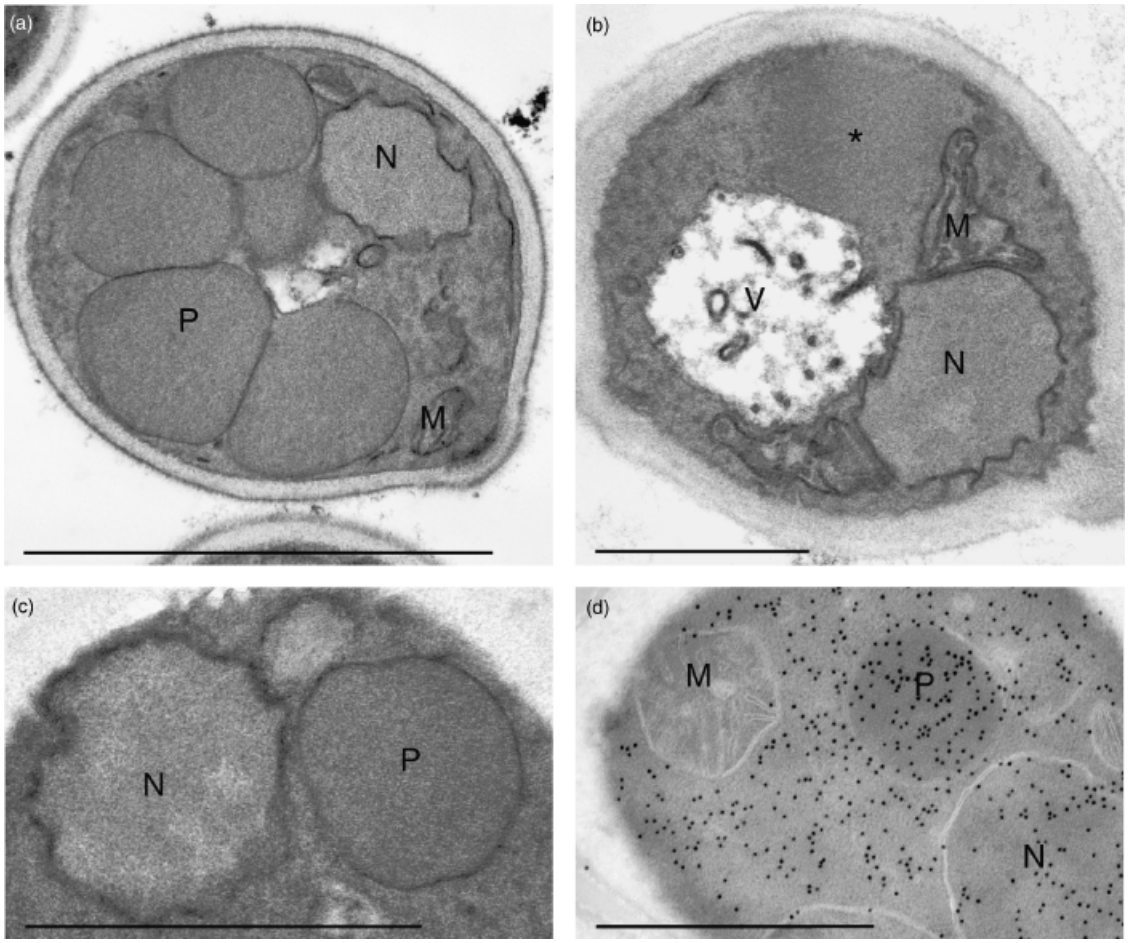

Fig. 6. Overproduction of *eEF1A* suppresses the peroxisome deficient phenotype of *H. polymorpha pex3-1* cells. (a–c) Morphology of a methanol-grown wild-type *H. polymorpha* cell containing many large peroxisomes (a) a methanol-induced *pex3-1* mutant cell, which completely lacks peroxisomal structures (b) and a section of a methanol-induced cell of *pex3-1* [pHIPX4-TEF2] demonstrating the presence of a single peroxisome in these cells (c). (d) Immunocytochemistry; a section of a cell of *pex3-1* [pHIPX4-TEF2] is shown that has been labeled using α-AO antibodies. Labeling is evident on the single peroxisome and the cytosol, but is absent in the mitochondrion (aldehyde, α-AO/GAR-gold, uranylacetate). Electron micrographs are taken of $KMnO_4$-fixed cells unless otherwise indicated. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. An asterisk indicates the location of a cytosolic AO crystalloid. The marker represents 0.5 μm unless indicated otherwise.

peroxisome formation, clearly implying that the *pex3-1* allele was essential for the suppression effect (data not shown).

Co-overexpression of the *pex3-1* allele and *TEF2* fully restores peroxisome biogenesis in a *pex3* strain

In order to obtain more information as to why eEF1A overproduction partly suppressed the peroxisome biogenesis defect in the *pex3-1* mutant, we decided to investigate this mutant in more detail. Sequencing of the mutant allele resulted in the identification of a G1925A mutation in the *PEX3* sequence (see Genbank U37763) that changed codon 243 from UGG (Trp) to UGA (Ter). Thus, the *pex3-1* allele could still produce a C-terminally truncated protein with a calculated Mr of 28 kDa. However, Western blot analysis failed to detect any protein of this size in crude extracts of *pex3-1* cells (Fig. 7a), suggesting that either the truncated protein or the mRNA encoding it may be unstable in the cell.

In eukaryotes, eEF1A plays a crucial role in translation elongation. However, additional roles for this protein (e.g. regulating translational fidelity or bundling of actin filaments) have also been proposed. We reasoned that upon eEF1A overproduction, the nonsense codon in *pex3-1* mRNA might be occasionally misread, resulting in the production of a minor amount of functional Pex3p. To test this possibility, we constructed *H. polymorpha pex3* strains that overexpressed two different versions of the mutant *pex3-1* allele. In the first strain (KEF2-1), only the protein-coding portion of the *pex3-1* allele (up to nt 1997 in

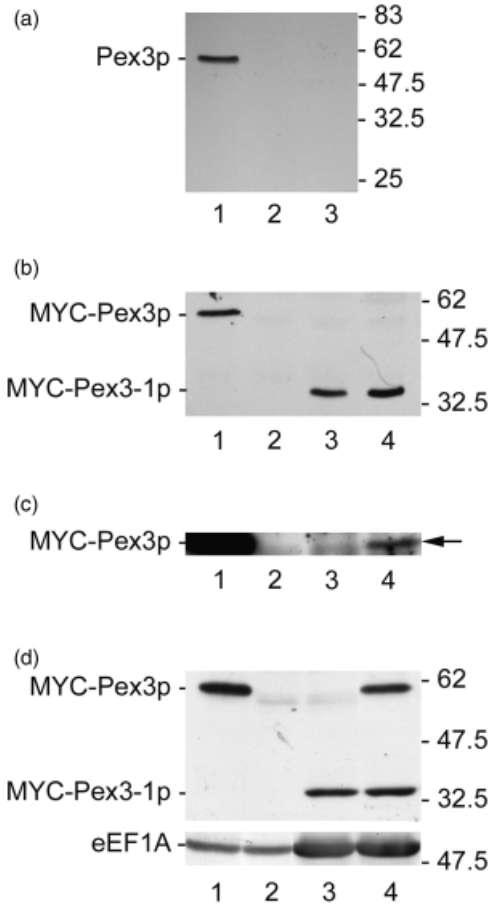

Fig. 7. Detection of mutant forms of *Pex3p*. (a) Western blot analysis of crude cell extracts of *H. polymorpha* wild-type (lane 1), *pex3-1* (lane 2) and *pex3-1* [pHIPX4-TEF2] (lane 3) cells. (b and c) Western blot analysis of *H. polymorpha pex3* [pRBG61] (lane 1), *pex3* (lane 2), KEF2-1 (lane 3) and KEF9-6 (lane 4) cells. (d) Western blot analysis of *H. polymorpha pex3* [pRBG61] (lane 1), *pex3* (lane 2), KEF2-1::P_{AOX}-*TEF2* (lane 3) and KEF9-6::P_{AOX}-*TEF2* (lane 4). In all cases, cells were grown on media containing methanol/glycerol/ammoniumsulphate to induce peroxisomes. Samples were taken after 20 h of growth and TCA precipitated. Subsequently, crude extracts were prepared and analysed by Western blotting using a specific polyclonal antiserum against Pex3p (a), a monoclonal antibody against the MYC epitope (b–d) or a polyclonal antiserum against eEF1A (d). Equal amounts of protein were loaded per lane. (c) Shows a section of a highly overexposed Western blot with the identical samples as shown in b (region of the blot containing MYC-Pex3p). The arrow points to the minor amount of MYC-Pex3p detected in crude extracts of strain KEF9-6. The molecular weights of marker proteins are indicated in kiloDalton to the right.

Genbank U37763) is present. The second strain (KEF9-6) overexpresses the entire *pex3-1* allele including the now untranslated portion of *PEX3*, thus allowing possible translational read-through. To facilitate detection of the proteins produced by the strains, both *pex3-1* derivatives were MYC-tagged at their 5′ ends. As a control, *H. polymorpha pex3* [pRBG61] was used which contains a 5′ MYC-tagged wild-type *PEX3* gene under the control of its endogenous promoter (Haan *et al.*, 2002).

Ultrastructural analyses of methanol-induced cells of the strains KEF2-1 and KEF9-6 revealed dramatic differences in their peroxisomal profiles. As shown before (Haan *et al.*, 2002), expression of *MYC-PEX3* by its endogenous promoter fully complemented the peroxisome biogenesis defect of the *pex3* strain and many large peroxisomes were formed in control cells (data not shown). In KEF2-1 cells, the production of the truncated MYC-tagged protein resulted in the formation of small membranous structures (Fig. 8a), which contained the peroxisomal membrane protein Pex14p (inset in Fig. 8b), but harboured only little matrix protein (Fig. 8b, shown for AO). KEF9-6 cells showed a heterogenous population in that some cells contained a few, very large peroxisomes which harboured most peroxisomal matrix proteins (Fig. 8c and d; shown for AO). In other cells only a single peroxisome was present and AO was located in both the peroxisome and a cytosolic crystalloid (Fig. 8e). Despite this partial restoration of the peroxisome biogenesis defect, KEF9-6 cells were unable to grow on methanol as sole carbon and energy source.

Biochemical analyses revealed that cells of KEF2-1 and KEF9-6 produced an *c.* 35-kDa protein that was recognized by the α-MYC antibodies (Fig. 7b). In both cases the amount of truncated protein produced by these overexpression strains approximately equalled that produced by the strain expressing wild-type *MYC-PEX3* by the much weaker endogenous promoter. Assuming that translational read-through of the *pex3-1* allele had caused the major ultrastructural differences observed between the two strains, some wild-type MYC-Pex3p should have been formed in KEF9-6 cells. Although such a protein band is not visible in Fig. 7b, a very long exposure of a similar Western blot demonstrated the presence of a minute amount of a protein of the expected size in extracts of KEF9-6 cells, which was absent in extracts of *pex3* and KEF2-1 cells (Fig. 7c).

To obtain definitive proof that eEF1A overproduction could result in translational read-through of the *pex3-1* allele, we also overexpressed *TEF2* in strains KEF2-1 and KEF9-6. Analysis of the growth characteristics of the resulting strains, KEF2-1::P_{AOX}-*TEF2* and KEF9-6::P_{AOX}-*TEF2*, demonstrated that eEF1A overproduction rescued the inability of strain KEF9-6 to utilize methanol as the sole source of carbon and energy, a hallmark of the presence of functional peroxisomes. In contrast, strain KEF2-1::P_{AOX}-*TEF2* remained unable to grow on methanol. Biochemical analyses of these strains revealed that KEF9-6::P_{AOX}-*TEF2* cells produced almost wild-type levels of full-length MYC-Pex3p in addition to the truncated MYC-Pex3-1 protein (Fig. 7d). In contrast, overproduction of eEF1A in strain KEF2-1 failed to result in any synthesis of full-length MYC-Pex3p.

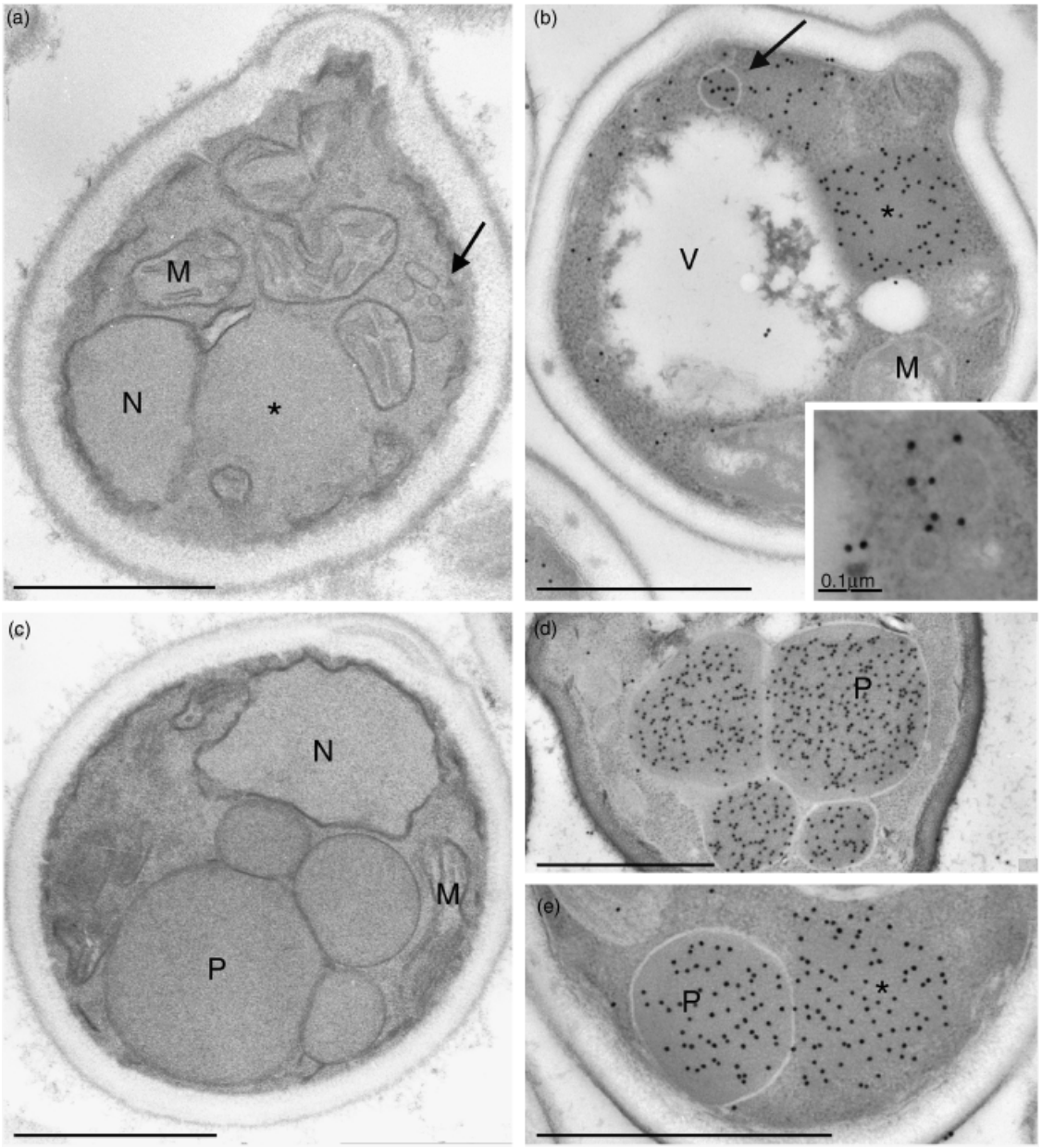

Fig. 8. Overexpression of the *pex3-1* allele restores peroxisome biogenesis in *pex3* cells via translational read-through. (a) Morphology of methanol/glycerol/ammoniumsulphate-grown KEF2-1 cell showing the small peroxisomal structures (*arrow*) induced by the production of MYC-Pex3-1p in *pex3* cells. (b) Immunocytochemistry showing that these structures (*arrow*) are only weakly labeled with specific antibodies against AO protein. Nevertheless, they contain the peroxisomal membrane protein Pex14p (inset in b), which is normally mislocalized to mitochondria in *pex3* cells (Baerends *et al.*, 2000). (c) Morphology of methanol/glycerol/ammoniumsulphate-grown KEF9-6 cell showing many large peroxisomes as a result of the formation of a small amount of full-length MYC-Pex3p by translational read-through of the *pex3-1* allele. (d and e) Immunocytochemistry of KEF9-6 cells with specific antibodies against AO protein showing that in some cells all AO label is confined to peroxisomes (d). Nevertheless, in other cells an AO label is present in peroxisomes, on a cytosolic AO crystalloid (*) and also in the cytosol, suggesting that overexpression of the full-length *pex3-1* allele does not completely complement the *pex3* strain (e). Electron micrographs are taken of $KMnO_4$-fixed cells unless otherwise indicated. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. An asterisk indicates the location of a cytosolic AO crystalloid. The marker represents 0.5 μm unless indicated otherwise.

Discussion

We have identified and cloned two genes encoding eEF1A in *H. polymorpha*. In this yeast, both genes are constitutively expressed. As expected, deletion of both genes was lethal. Deletion of the *TEF1* gene resulted in reduced growth on media containing methanol and D-alanine. Our data demonstrate that under these cultivation conditions, the

expression of both *TEF* genes is relatively low. We presume that upon deletion of *TEF1*, the gene with the highest expression, the remaining level of eEF1A in the cells is not sufficient to fully support a normal growth rate.

Furthermore, we found that eEF1A overproduction suppressed the peroxisome deficient *pex3-1* mutant that contains a UGG to UGA mutation in *PEX3*, resulting in premature translation termination. A similar suppression was also observed upon overexpression of the *pex3-1* allele in *pex3* cells, while additional co-overproduction of eEF1A in this strain restored the ability of the cells to utilize methanol as the sole carbon and energy source. Our data indicate that the partial suppression of the peroxisome biogenesis defect in the *pex3-1* mutant upon eEF1A overproduction was presumably not caused by a direct interaction between eEF1A and the truncated *pex3-1* gene product. Rather, we presume that the increased eEF1A levels caused enhanced translational read-through of the stop codon in the *pex3-1* mRNA, resulting in the formation of a minute amount of full-length Pex3p, which nevertheless remained below the limit of our detection. This assumption was confirmed by the observation that significant amounts of full-length MYC-Pex3p were present in a strain co-overproducing eEF1A and MYC-Pex3p(Trp243Ter). Such a suppression phenomenon is consistent with observations made in other yeast species regarding a role for eEF1A in translational fidelity (Song *et al.*, 1989; Silar & Picard, 1994; Silar *et al.*, 2000). Previously, we have observed a direct relation between the level of Pex3p and the number of peroxisomes in *H. polymorpha* (Baerends *et al.*, 1996). In line with this is the observation that eEF1A overproduction in *pex3-1* cells only allows the formation of a single peroxisome per cell. Recently, eEF1A was also isolated as a suppressor of the *Yarrowia lipolytica pex1-1 (pay41)* mutant, which is affected in peroxisome biogenesis and unable to grow on oleate as the sole carbon and energy source (V.I. Titorenko, unpublished data). It is tempting to assume that also in this case read-through of a stop codon has resulted in the formation of sufficient amounts of wild-type Pex1p to allow growth on media containing oleate.

The current analysis was initiated by the finding that a portion of eEF1A copurifies with peroxisomes (Figs 2 and 3). A number of recent proteomics studies on highly purified peroxisomes confirm that eEF1A associates with these organelles (see Schäfer *et al.*, 2001; Marelli *et al.*, 2004; our unpublished data). Several explanations could possibly clarify this phenomenon. eEF1A is a rather basic protein (pI = 9.1), which might bind rather nonselectively to negatively charged membranes. Alternatively, eEF1A may perform a specific function at peroxisomes e.g. in localized translation (Condeelis, 1995; Gonsalvez *et al.*, 2005). Currently, our data do not allow us to discriminate between these possibilities.

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